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14. ABSTRACT In the bone marrow from prostate cancer patients with clinical evidence of bone metastasis, we identified katanin p60 as a differentially expressed factor. Our preliminary studies showed that katanin p60 is expressed in prostate cancer cell lines and also in the prostate cancer tissues of local disease and bone metastasis, suggesting that katanin p60 is associated with prostate cancer progression and metastasis in bone. We hypothesize that katanin p60 serves as a cell migration factor to mediate prostate cancer metastasis to bone. In the proposed study, we will focus on the characterization of the katanin p60 in vitro and in vivo with regards to its functions in prostate cancer bone metastasis. Our proposed study is highly relevant to prostate cancer because the katanin p60 is apparently associated with osseous metastasis. Understanding the functional mechanism of katanin p60 in prostate cancer bone metastasis will provide opportunities for therapeutic intervention. Katanin p60 can be a novel therapeutic target for prostate cancer bone metastasis, or a biomarker for early diagnosis. Therefore, the outcome of this study will help us to reduce prostate cancer related mortality.

15. SUBJECT TERMS
prostate cancer, metastasis, katanin, cell motility

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Role of Katanin in Prostate Cancer Bone Metastasis

Introduction

The focus of this proposed study is to understand the mechanisms leading to progression of prostate cancer in bone. Prostate cancer is the second most common cause of cancer-related death among men in the United States. The late stage of androgen-refractory prostate cancer is dominated by complications arising from bone metastasis[1]. To date, there is no effective treatment for bone metastases.

Our early study using proteomics approach has identified a low molecular weight katanin p60 isoform in the bone marrow samples from prostate cancer patients with clinical evidence of bone metastasis. Katanin p60 is a member of AAA (ATPases associated with various cellular activities) protein family and has a microtubule-severing activity. Its biological functions have been known for involving in cell mitotic division[2-4] and neuronal migration[5-7]. However, the katanin p60 isoform or its role in the cancer metastasis has never been reported. In this study, we will characterize katanin p60 and its isoforms *in vitro* and *in vivo* with regards to its functions in prostate cancer bone metastasis.

In the previous report period, we have identified several alternative splicing forms of katanin p60 (KATNA1) in prostate cancer samples. Based on structural information, these isoforms contain the same N-terminal part but with different alterations in the middle segment or at C-terminus. The conservation in the N-terminal sequence led us to predicate these alterations may not affect the protein-protein interactions with the N-terminal binding partner, katanin p80, which serves as a scaffold to bring katanin p60 to centrosome. These alterations, however, may render the changes in the ATPase activity and subsequently affect on the microtubule-severing and cellular activity.

In this report, we focused on one of the katanin p60 isoforms, KATNA1-ΔE5-7, because we found it was expressed only in the prostate tissue samples with various disease stages but not in the normal prostate. We also describe our research progress in

the generation of several antibodies for tissue profiling as proposed in Statement of Work (outlined below). The outcome of the study will help us to understand the mechanism of katanin-mediated cellular activity and to find relevant targets for prostate cancer therapy.

- Assessment of the effects of katanin p60 on cell motility by RNA interference. (months 13-15)
- Generate and characterization of peptide antibodies against an alternative splicing form of katanin p60. (months 3-9)
- Profiling of the expression and localization of katanin p60 in the tissue samples at various stages of prostate cancer. (months 16-24)

Report Body

We have cloned and sequenced several alternative splicing transcripts of the katanin p60 gene (KATNA1) from human prostate samples. The most frequently detected isoform was KATNA1-ΔE7, in which the exon 7 sequence is excluded in the mature mRNA transcript (**Fig. 1B-a**). The isoform protein is expected to reduce the molecular mass of katanin p60 from 56 kDa to 50 kDa.

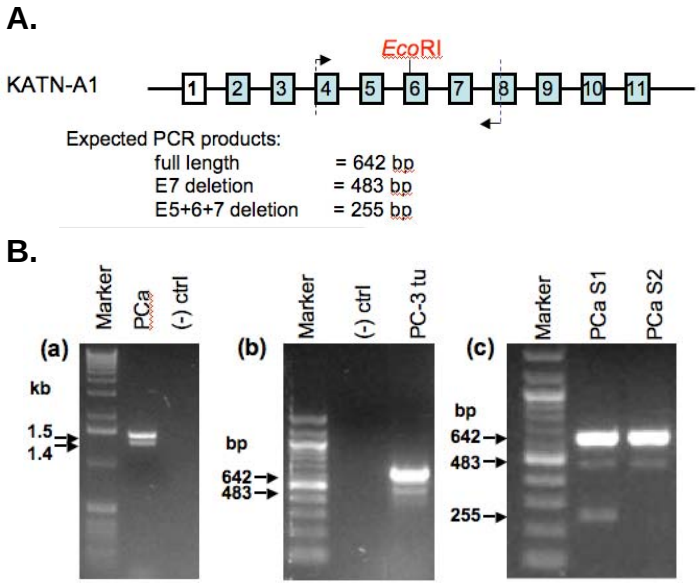

Fig. 1. RT-PCR of katanin p60. ***A.*** PCR primers used in detection of alternative splicings shown in panel B (b) and (c). ***B.*** **(a)** RT-PCR of a prostate cancer sample (PCa). The amplicons have been confirmed to be the full-length KATNA1 and KATNA1-ΔE7. **(b)** RT-PCR of a PC-3 xenograft tumor. **(c)** RT-PCR of prostate cancer samples. The amplicons were cloned and sequenced, which are KATNA1, KATNA1-ΔE7 and KATNA1-ΔE5-7, respectively.

Another frequently detected isoform in the prostate cancer samples was KATNA1-ΔE5-7 that skips the exon 5, 6 and 7 of KATNA1 in the mature transcript (**Fig. 1B-c**). The predicated molecular size of this isoform is 41 kDa, which is very close to the low molecular weight katanin protein (~45 kDa) that we identified in proteomics. The distributions of these two katanin p60 isoforms in human prostate tissues were then analyzed with TissueScan Prostate Cancer Panel I (Origene, Rockville, MD), we found that KATNA1-ΔE7 was expressed broadly in most of samples including the normal ("Stage 0") and the ones with prostate cancer, while the KATNA1-ΔE5-7 was not expressed in the normal samples, but was only expressed in some samples with benign prostate hyperplasia (BPH) and with the prostate cancer from the disease stage II to stage IV (**Fig. 2**). The result suggests that expression of the KATNA1-ΔE5-7 isoform is relevant to the disease progression of prostate cancer.

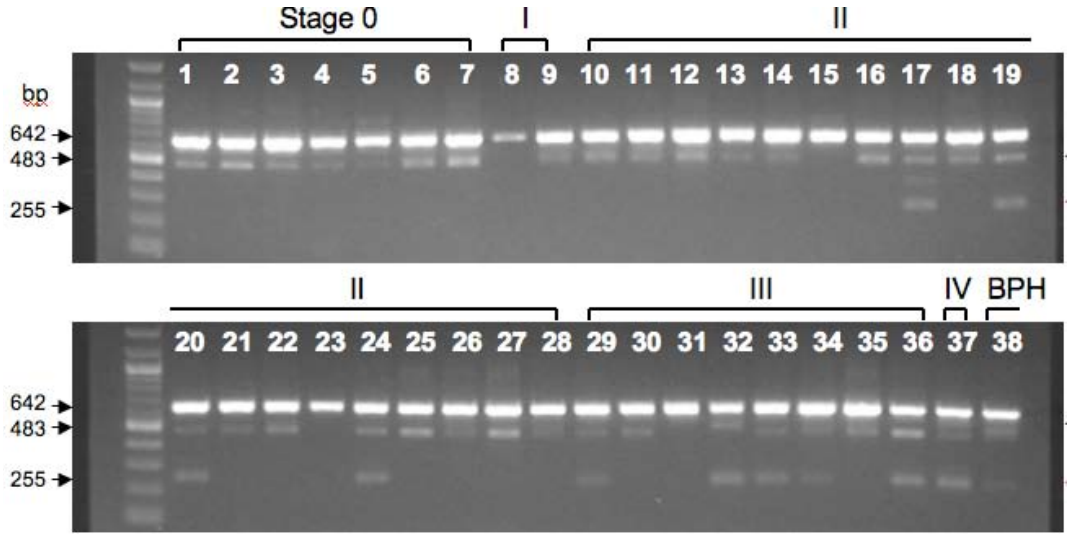

Fig. 2. Analysis of TissueScan Prostate Cancer Panel I by RT-PCR. Total 38 individual samples were shown. The 642 kb bands represent the KATNA1 transcripts with inclusion of exon 5, 6 and 7. The 483 kb bands were derived from the transcript that skips exon 7; while 255 kb bands were from the transcripts excluding the exon 5, 6 and 7.

We had proposed to use RNA interference for knocking down KATNA1 and to assess the cellular responses (see outlined Statement of Work). With a pool of four KATNA1-specific shRNAs (SHDNA-NM_007044, Sigma-Aldrich) that respectively

target the N-terminal or C-terminal part of KATNA1, we achieved >50% reduction of katanin p60 protein in some batches of cells (**Fig. 3b**). However, we did not observe any

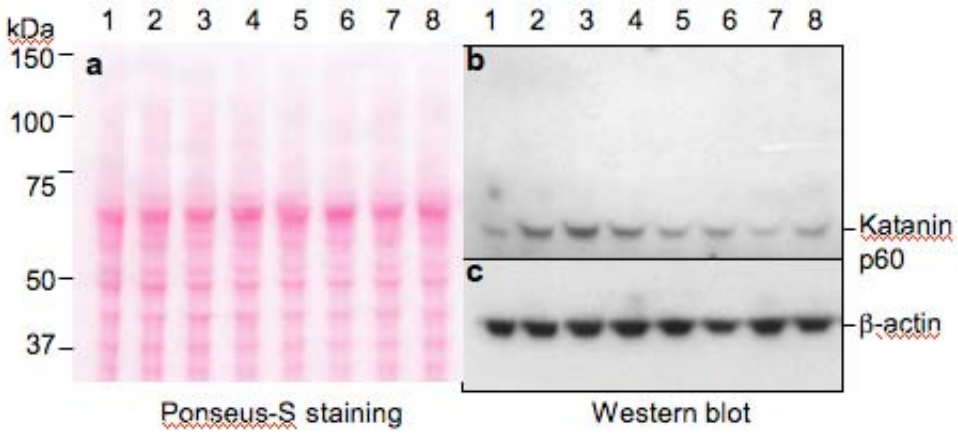

Fig. 3. Knockdown of KATNA1 by shRNA. (**a**) Total protein staining. (**b**) Western blot of katanin p60 with the K1 antibody. (**c**) β-actin as loading control. The numbers indicate individual samples from different shRNA combinations. Number 1, 5 and 7 showed >50% reduction.

significant alteration of the cell properties *in vitro* (data not shown). This could be due to either the insufficient knockdown for change of the cellular activity or a possible compensatory effect by KATNA1-like gene(s) in cells. On the other hand, we learn from the analysis of TissueScan Prostate Cancer Panel that total mRNA level of KATNA1 was not significantly different between the normal samples and the disease samples. However, the expression of the alternative splicing KATNA1-ΔE5-7 was significantly increased in disease samples. The KATNA1-ΔE5-7 isoform may have an unusual function to the cancer cells in prostate tissue. We have checked the KATNA1-ΔE5-7 expression in several prostate cancer cell lines by RT-PCR and found that the *in vitro* cultured cells only express low level of KATNA1-ΔE5-7 (data not shown). Thus, the shRNA-mediated knockdown will not enhance the KATNA1-ΔE5-7 effect in these cells. Instead, we should approach the problem by overexpression of KATNA1-ΔE5-7 in the prostate cancer cells.

We have cloned the KATNA1-ΔE5-7 gene from a prostate cancer cDNA pool with high fidelity DNA polymerase *pfu* Ultra™ Hotstart (Stratagene). The gene was initially inserted in pcDNA3.1D/V5-His vector (Invitrogen) and confirmed by DNA

sequencing. In order to study the cellular localization and function of KATNA1-ΔE5-7, we subcloned the gene into a pEGFP-N3 vector to produce the pEGFP-KATNA1-ΔE5-7 fusion construct; meanwhile, we also made pEGFP-KATNA1 and pEGFP-KATNA1-ΔE7 constructs for comparison. We used these plasmid constructs in transient transfection assays. Western blot analyses of the transient transfected PC3 cells showed that KATNA1-ΔE5-7-EGFP fusion protein was expressed at a detectable low level in PC3 cells (**Fig. 4**).

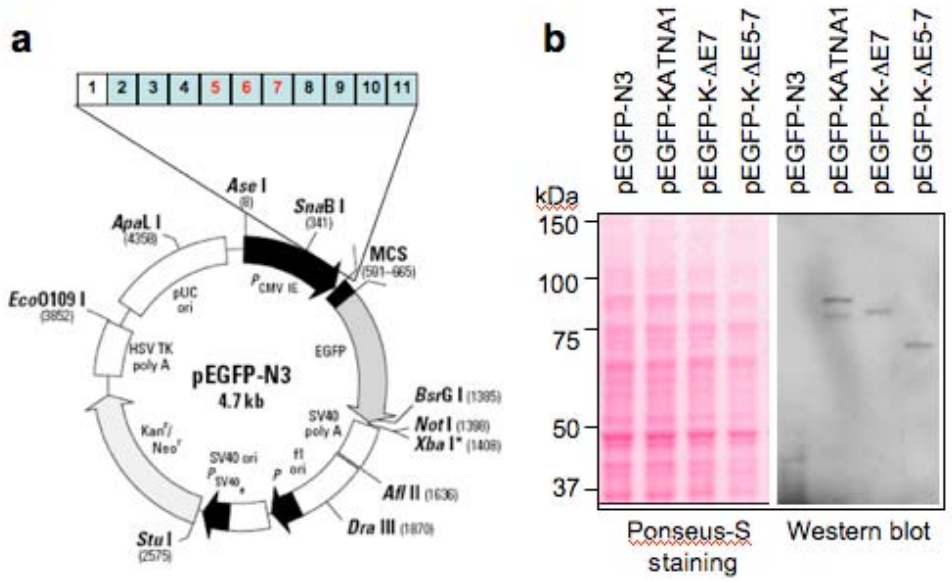

Fig. 4. Expression of KATNA1-ΔE5-7 in PC3 cells. (**a**) Diagram of constructs for expressing katanin p60/isoform-EGFP fusion protein. (**b**) Western blot with anti-GFP antibody showed KATNA1-ΔE5-7 and other controls were expressed with expected protein size. The KATNA1-ΔE5-7-EGFP is about 65 kDa.

We followed up the pEGFP-KATNA1-ΔE5-7 transient transfection to examine the cell morphological changes. The KATNA1-ΔE5-7-GFP-positive cells showed a slightly depolarized phenotype (**Fig. 5**). However, because of very low transfection efficiency (tested with multiple different methods) we were not able to quantitatively assess the cellular responses. In order to increase the transfection efficiency, we are currently in process to make viral delivery of the KATNA1-ΔE5-7 and control genes to prostate cancer cells. We have subcloned the KATNA1-ΔE5-7 into a retroviral vector pBMN-IRES-GFP (a kindly gift from Dr. Gary Nolan, Stanford University) to produce pBMN-K(ΔE5-7)-IRES-GFP. We are currently preparing a batch of recombinant

retrovirus with pBMN-K(ΔE5-7)-IRES-GFP for viral infection of LNCaP and PC3 cells. The cellular functional study of KATNA1-ΔE5-7 in these prostate cancer cells will be performed subsequently.

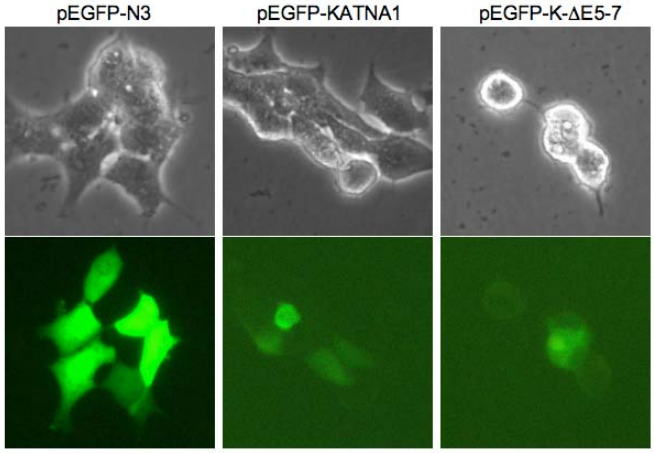

Fig. 5. Morphology of the transiently transfected cells. The cells were transfected with pEGFP-N3-derived plasmid DNA for 24 hours. Cell images were taken from the culture plates under fluorescent microscope. Note that pEGFP-K-ΔE5-7 transfected cells showed a depolarized phenotype.

While we carried out the studies of the katanin p60/isoforms in prostate cancer cell lines *in vitro*, we also started to examine the expression and localization of katanin p60/isoform *in vivo*. One of the key reagents for the *in vivo* studies is the specific antibody. There was no commercially available anti-katanin p60 antibody until Abnova released a polyclonal antibody MaxPab-KATNA1 (B01P) in late 2008. Our immediate test indicates B01P does react with recombinant katanin p60, but it also cross-reacts with some high molecular weight, non-specific proteins in cell lysates. Far before the B01P antibody coming to market, we had put in a tremendous effort to produce our own customized antibodies for detection of katanin p60. We had prepared a large amount of polyhistidine-tagged human katanin p60 from insect cell culture and purified the recombinant protein with Ni-NTA agarose column. The purified recombinant katanin p60 protein was then used for generating polyclonal and monoclonal antibodies. One of our successful antibodies specific to katanin p60 is a rabbit polyclonal antibody named K1. In Western blotting, the purified K1 not only reacts to the his-tagged recombinant katanin p60 protein but also reacts to the endogenous 56 kDa katanin p60 in DU145 cell lysate (**Fig. 6a**). Similarly, it reacts strongly to the 56 kDa katanin p60 in the 293 cell lysates. In addition to the dominant katanin p60 band, the K1 antibody detects some low molecular weight bands in different cells, such as a ~40 kDa in DU145 and a ~30 kDa in the 293 cell (**Fig. 6a**), which perhaps are the isoforms or the degradation products of katanin p60.

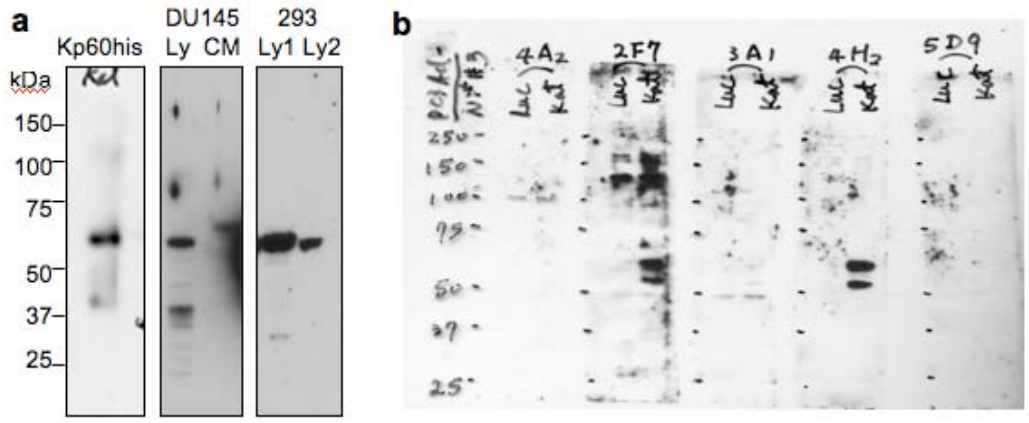

Fig. 6. Analyses of anti-katanin p60 antibodies. (**a**) The K1 antibody-mediated detection of katanin p60 protein. Kp60his: the polyhistidine-tagged katanin p60 protein fraction; Ly: cell lysate; CM: conditioned medium. (**b**) Immuno-precipitation and Western blot testing of monoclonal katanin p60 antibodies. Luc: the protein fraction from a negative control of adenoviral (Adluc) infection; Kat: the protein fraction from positive control of adenoviral (AdKat) infection.

In addition to the K1 antibody, another rabbit polyclonal antibody, named K2, also positively reacts to katanin p60 in cell lysate but with higher background in Western blot (data not shown). We have yet purified the K2 for further testing. Moreover, we have generated and tested five independent mouse monoclonal antibodies from the primary screening. They were named K-2F7, K-3A1, K-4A2, K-4H2 and K-5D9 respectively. The test in immuno-precipitation and Western blotting, we found K-2F7 and K-4H2 bound effectively to the recombinant katanin p60 protein (**Fig. 6b**). These katanin p60-specific antibodies are potentially very valuable for the mechanistic research and potentially for clinical application in future.

As a part of our proposed studies to understand the relationship between katanin p60 and prostate cancer, we investigated the expression and localization of katanin p60 in the tissue samples of prostate cancer. Immunohistochemical staining of the tissue sections was performed with the purified K1 antibody and a HRP-conjugated secondary antibody. Results showed that katanin p60 proteins (the K1 antibody may not distinguish the full-length from isoforms) are expressed relatively high in the prostate epithelial compartment but very low in stromal compartment (**Fig. 7a**). The difference of katanin p60 distribution between these two compartments is much obvious in the tissue samples with high grade PIN (prostatic intraepithelial neoplasia) (**Fig. 7b**). The positive staining of katanin p60 proteins is also shown in the samples of bone metastasis (**Fig. 7c**) and the brain

metastasis (**Fig. 7d**) of prostate cancer. The subcellular localization of katanin p60 proteins is in the paranuclear cytoplasma of prostate epithelial cells; whereas it is much diffused in the cytoplasma of cancer cells in bone metastasis (**Fig. 7c**), but is condensed more in the nuclei of cancer cells in brain metastasis (**Fig. 7d**). These observations suggest that expression and subcellular localization of katanin p60 proteins are possibly changed during disease progression, and also likely influenced by local pathophysiology and tumor microenvironment.

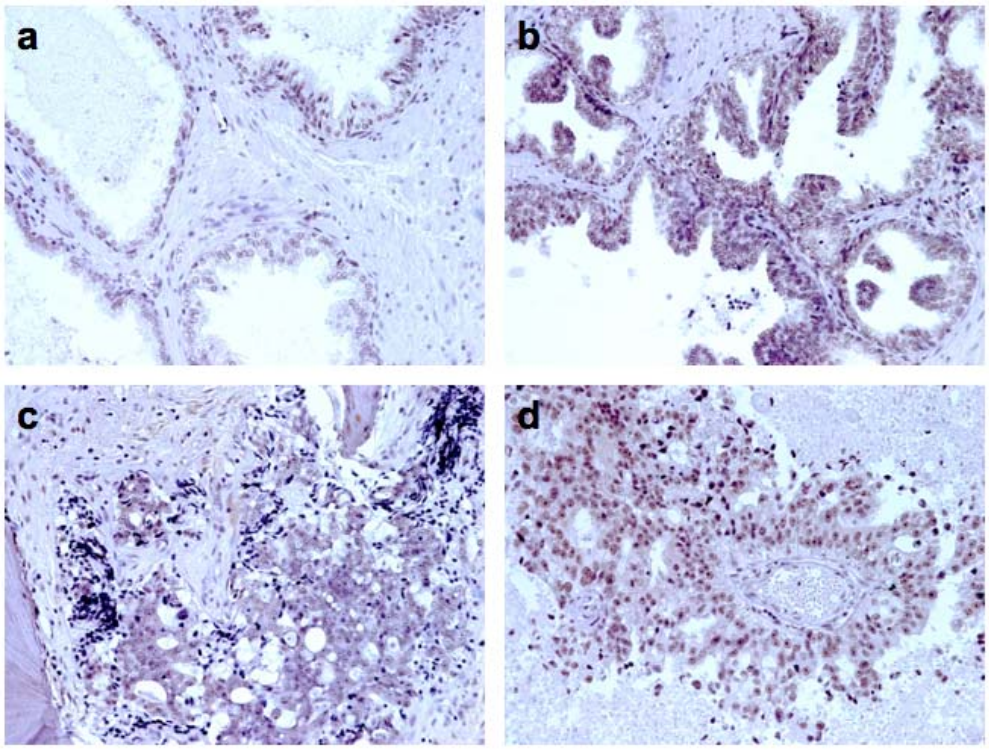

Fig. 7. Immunohistochemical staining of prostate cancer samples with the K1 antobody. (**a**) Normal prostate. (**b**) High grade PIN (prostate intraepithelial neoplasia). (**c**) Bone metastasis from prostate cancer. (**d**) Brain metastasis from prostate cancer.

Since we lately identified the KATNA1-ΔE5-7 isoform as an emerged molecular candidate correlating to the disease progression of prostate cancer (**Fig. 2**), we think that the positive stainings by the K1-mediated immunohistochemistry in the prostate cancer samples might reflect the total signals of katanin p60 and isoforms. The K1 antibody could not distinguish the p60 from the isoforms. Thus, we designed a peptide called Ke37 that embraces the katanin p60's exon 4 to exon 8 splicing junction for producing the

customized KATNA1-ΔE5-7 specific antibodies. Recently, we obtained polyclonal antibody sera from two peptide-conjugates immunized rabbits, named Ke37-1 and Ke37-2. In Western blot testing the antibody sera were able to positively react with the 65 kDa KATNA1-ΔE5-7-EGFP fusion protein in cell lysates (**Fig. 8a, b**). However, the antibody sera also cross-reacted with many non-specific proteins in cell lysates, suggesting that the sera must be processed by affinity purification with the Ke37 peptide or the recombinant KATNA1-ΔE5-7 protein in order to reduce the cross-reactions. Recently, we have tested a batch of Ke37-2 serum partially purified by the peptide-mediated affinity purification. The result showed it yielded cleaner background but still with multiple bands in Western blot (**Fig. 8c, d**). One of strong immunoreactive band is about 40 kDa, which is the predicted size of endogenous KATNA1-ΔE5-7 protein, suggesting that the Ke37-2 antibody could interact with KATNA1-ΔE5-7 protein and potentially very useful for investigation of this specific katanin p60 isoform in prostate cancer samples.

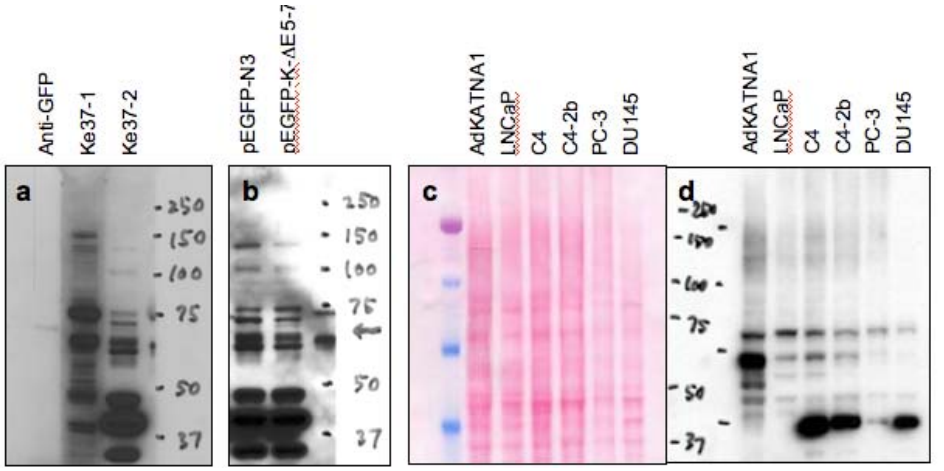

Fig. 8. Examination of the Ke37 peptide antibodies. (**a**) Test of the Ke37 antibodies in Western blotting of a cell lysate containing KATNA1-ΔE5-7-GFP fusion protein. (**b**) Test of Ke37-2 antibody in Western blotting of cell lysates with or without KATNA1-ΔE5-7-GFP fusion protein. (**c**) Total protein staining. (**d**) Test of Ke37-2 in various PCa cell lysates.

While in the process to further optimize the antibodies, we have tested the partially purified Ke37-2 antibody in parallel to the K1 antibody in immunohistochemical staining of few sections of prostate tissue. The result showed that the Ke37-2 antibody yielded no visible staining in most area of tissue section, but produced weak staining in few isolated foci (**Fig. 9a**). In contrast, the K1 antibody produced more intensified

stainings, particularly in the glands with hyperplasia (**Fig. 9b**). We also noticed that the Ke37-2 positive staining appeared to be more nuclear localization than that of the K1 positive staining. Further study will be performed with the optimized antibdodies in a broad spectrum of prostate cancer tissue samples, which will be able to determine the relationship between the KATNA1-ΔE5-7 expression and the prostate cancer progression.

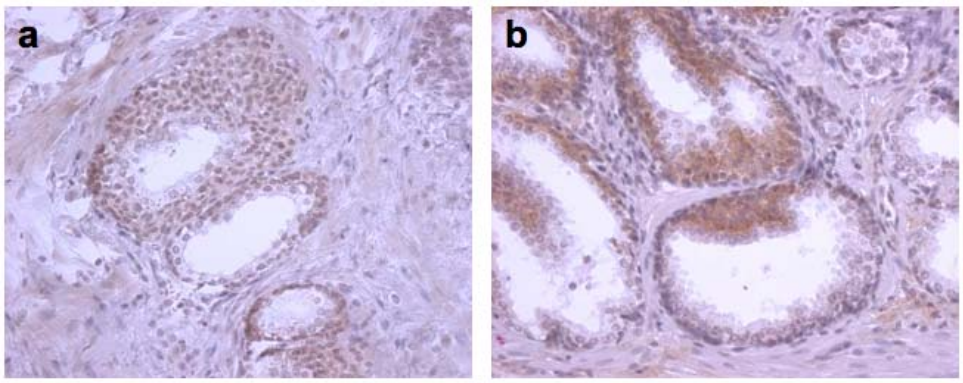

Fig. 9. Immunohistochemical staining of prostate tissue sections. (**a**) Staining with the partially purified Ke37-2 antobody. One of positive stained foci was shown. (**b**) Staining with the purified K1 antibody.

Key Research Accomplishments

- Identified the katanin p60 isoform KATNA1-ΔE5-7 in correlation with the prostate cancer disease progression.
- Cloned the KATNA1-ΔE5-7 cDNA for molecular mechanistic studies.
- Achieved shRNA-mediated katanin p60 knockdown in the *in vitro* study.
- Generated two polyclonal and five monoclonal anti-katanin p60-specific antibodies, and established the K1 antibody as a reliable primary antibody for detection of katanin p60 in cell lysates and tissue sections.
- Generated two polyclonal peptide antibodies against the katanin p60 isoform KATNA1-ΔE5-7. The partially purified Ke37-2 antibody showed promisingly in detection of the specific katanin p60 isoform.

- Characterized the expression and localization of KATNA1/isoform in the prostate cancer tissue samples.

Reportable Outcome

The KATNA1 and novel katanin p60 isoform KATNA1-ΔE5-7 cDNA clones, expression plasmids, viral reagents, and antibodies have been produced. However, the properties of these products need to be further characterized and optimized. Manuscripts are in preparation for publication.

Conclusion

We have identified a novel katanin p60 isoform KATNA1-ΔE5-7 in prostate cancer samples. Importantly, this isoform is undetectable in normal prostate but is often increased during prostate disease progression. We have cloned this isoform gene and made new constructs for overexpression in prostate cancer cells. The transient transfection to deliver the recombinant KATNA1-ΔE5-7 gene appears to affect the epithelial cell polarity, suggesting that this isoform may have a dominant effect or an unusual function in the regulation of cancer cell activities.

In order to examine the expression and localization of katanin p60 and isoform proteins in prostate tissues, we generated and tested several antibodies. The purified and validated K1 antibody reacts specifically and strongly to the 56 kDa katanin p60 protein. It also detects some minor, low molecular weight species including a ~40 kDa protein (similar to the predicated size of KATNA1-ΔE5-7) in cell lysates. Use of the K1 antibody for immunostaining of prostate tissue samples, we found that expression of katanin p60/isoform was readily detectable in basal cell hyperplasia, cancerous and metastatic cells, but the subcellular localization of katanin p60/isoform varied, perhaps due to the cellular response to disease progression or tumor microenvironmental changes.

In additions, we have generated a peptide antibody Ke37-2 for detection of the unique splicing junction site in KATNA1-ΔE5-7. This antibody reacts both the KATNA1-ΔE5-7 recombinant protein and a ~40 kDa endogenous band in cell lysates.

We will further verify and purify this antibody for *in vivo* studies. Meanwhile, we have tested a partially purified Ke37-2 antibody in immunostaining of prostate tissues. The result showed the antibody had a limited reaction with few foci in the tissues, suggesting that KATNA1-ΔE5-7 is generally suppressed in normal glands. Emergence of this isoform may change the katanin p60 activity and cause the microtubule cytoskeleton reorganization in cancer cells. Thus, the final outcome of this study will help us to understand the function of katanin p60 isoform in cancer cells and to find relevant targets for cancer therapy.

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Appendices

None.